Title: Insights into the ropy phenotype of the exopolysaccharide-producing strain *Bifidobacterium animalis* subsp. *lactis* A1dOxR

Running title: Ropy phenotype in *B. animalis* subsp. *lactis*

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Abstract

The proteome of the ropy strain *Bifidobacterium animalis* subsp. *lactis* A1dOxR, compared to its non-ropy isogenic strain, showed an over-production of a protein involved in rhamnose biosynthesis. Results were confirmed by gene expression analysis and this fact agreed with the higher rhamnose content of the ropy exopolysaccharide.

The ability to synthesise an extracellular layer of carbohydrates, or exopolysaccharides (EPS), is a common trait in bacteria such as those members of the microbiota inhabiting our gut (1, 2) including *Bifidobacterium* and *Lactobacillus* (3, 4, 5). For instance, some strains of these human commensal genera are able to produce complex EPS under laboratory conditions. These polymers, built on repeating units of different monosaccharides, are also known as heteropolysaccharides (6, 7). Recently, EPS-producing bacteria have received particular attention due to the presumptive implication of the polymers in the cross-talk between bacteria and host (8, 9, 10, 11). However, there is limited information as to why only some specific EPS are able to interplay with human cells (12, 13, 14). Previous results from our group, also supported by other literature data, have shown that some physicochemical characteristics of EPS could be correlated with their immune modulating capability. Specifically, it seems that polymers having high molecular weight (HMW) are able to act as suppressors of the immune response (15). Part of the results supporting this hypothesis were obtained with a model of three isogenic EPS-producing *Bifidobacterium animalis* subsp. *lactis* strains A1, A1dOx and A1dOxR, the latter being able to synthesised a HMW-EPS fraction with immune-suppressive profile (13).

This strain A1dOxR, also named IPLA-R1, displays a characteristic "ropy" phenotype, denoted by the formation of a long filament when a loop is introduced into the colony. Interestingly, this ropy character was not observed in the two related (isogenic) EPS-producing strains, A1 and A1dOx, which also lacked the production of the HMW-EPS fraction; whereas, it seems that all three strains synthesise a low molecular weight (LMW)-EPS fraction (Table 1). Additionally, the monosaccharides building the EPS produced by the three strains are the same (glucose, galactose and rhamnose) but they are present at different ratio (16). Furthermore, the LMW-EPS from the two isogenic strains did not elicit a suppression of the immune response (13). Therefore, it seems that both, the capability to suppress the immune response and the ropy phenotype in strain A1dOxR, may be related with the synthesis of the HMW-polymer. In this regard, it has been indicated that the capability of certain EPS-producing bacterium to confer ropiness to a fermented product is directly

related to the molecular weight of its polymer. That is, ropy strains have EPS of high molecular weight whereas non-ropy strains produce polymers of smaller molecular weight (17, 18). In the current work, we have tried to gain insight into some molecular and physiological aspects of the occurrence of the ropy phenotype in the strain *B. animalis* subsp. *lactis* A1dOxR.

The strains A1 (parental, isolated from a fermented dairy product), A1dOx (adapted to OxGall by exposure of strain A1 to increasing concentrations of these bile salts) and A1doxR (derivative of A1dOx which, after consecutive generations in the absence of bile spontaneously acquired a ropy phenotype) were grown in MRSC [MRS (Difco, BD Biosciences, San Diego, CA) containing 0.25% L-cysteine (Sigma Sigma-Chemical Co., St. Louis, MO)] as previously described (13). As an initial approach, several physiological parameters were determined during the growth of the strains in order to denote potential differences among them. Growth curves were performed for 24 h at 37°C under anaerobic conditions in, at least, two biological replicates and measurements in each replicate were done in duplicate (see supplementary material). The evolution of OD_{600nm} and counts was parallel in the three bifidobacteria and statistical differences were not detected in the specific growth rate (μ_{max}) among the strains (μ_{max} = 0.58±0.01, 0.57±0.02 and 0.56±0.01 for A1, A1dOx and A1dOxR, respectively). Besides, the acidification rate, determined by monitoring the pH decrease and by analysing the production of total (acetic + lactic + formic) organic acids, was similar in the three strains. The final values of these parameters reached after 24 h of incubation are collected in Table 1. Regarding the EPS synthesis, the expression levels of the two priming-glycosyltranferase genes (*cpsD* and *rfbP*) located within the *B. animalis* subsp. *lactis eps* cluster did not show any significant variation among the three strains (Figure 1A). This suggests that the amount of EPS synthesised by the three strains was rather similar. Indeed, the production of total EPS, quantified by means of fluorescent-conjugated lectins (19), showed slight differences among the three strains (Figure 1B). This variability could be related with the different sensitivity of this lectin, which is specific for the detection of α-mannopyranosyl and α-glucopyranosyl residues, to detect the monosaccharides present at different ratio in the three polymers (16). Nevertheless, all

previous facts suggest that the glucose turnover in metabolic pathways, such as the glycolysis and the EPS biosynthesis, was not significantly different among the three strains and, therefore, it could not explain the occurrence of the ropy phenotype of the strain A1doxR.

In a step forward, we also wanted to check whether differences at molecular level could be detected among the three isogenic strains. For this purpose, a proteomic analysis was carried out from samples collected in the same growth phase (~6 h, OD_{600nm} ~1). Cultures were broken by sonication and proteins from the cell-debris free supernatants were precipitated by methanol-chloroform (3:1, vol/vol) and finally frozen until use (see supplementary material). The protein extracts were analysed by means of 2D-difference in gel electrophoresis (2D-DIGE) comparing the strain A1 *vs.* A1dOx and A1dOx *vs.* A1dOxR . The proteomes of the non-ropy strains (A1 and A1dOx) did not show apparent differences (data not shown); however, the comparison of A1dOx with its ropy-derivative A1dOxR evidenced a few proteins that differed between them (Figure 2A). One of these proteins (spot R3) was present in the extracts of the strain A1dOxR, but not in those of A1dOx; it was annotated in the NCBI protein database as the dTDP-D-glucose 4,6-dehydratase (RfbB, COG1088) and it is involved in the biosynthesis of L-rhamnose in Gram-negative bacteria (20). Remarkably, this enzyme is encoded by the first gene (Balat_1378) out of the three detected for this biosynthetic pathway inside the *eps* cluster in *B. animalis* subsp. *lactis* (Figure 2B). Then, specific primers (see supplementary table S1) were designed to check the expression of these three genes by quantitative reverse transcriptase-PCR in our strains. From this analysis, an over-expression of 5.01±0.62, 4.57±1.2 and 7.16±1.17 folds for Balat_1376, Balat_1377 and Balat-1378 genes, respectively, was detected in the ropy A1dOxR strain with respect to its parental A1dOx. A similar level of over-expression was detected in the two intergenic regions of the cluster (5.60±0.25 and 8.61±0.41 for intergenic regions Balat_1376-1377 and Balat_1377-1378, respectively), suggesting an operon structure. Thus, it seems that the rhamnose biosynthetic pathway was activated in the strain A1dOxR. Indeed the monosaccharide composition, determined by GC-MS technology as previously described (7), of the EPS purified from both strains showed a higher

percentage of rhamnose in the polymer A1dOxR (34.6±2.6) with respect to that detected in the polymer A1dOx (24.3±0.8). This fact could also explain the high (50%) rhamnose content found in the repeating unit structure of the HMW-EPS fraction synthesized exclusively by the ropy A1dOxR strain, which was determined in a previous work by NMR (21). The three genes of the rhamnose biosynthesis present in the *eps* cluster and the flanking regions of this cluster were sequenced in the three strains, but no SNPs were detected among them. This suggests that a pleiotropic effect of a mutation(s) located somewhere else in the chromosome, or a loss in the transcriptional control of the operon, may be on the basis of changes in expression of these genes in the ropy strain. On the other hand, a second protein (spot V2, Figure 3A) showing homology to the DNA-binding protein HB1 was detected only in the non-ropy strain A1dOx. This protein belongs to the DNA-BII family and is able to bind and bend DNA, thus acting as an architectural factor that could mediate in many cellular processes (22). No variations in the sequence of the corresponding gene, or in its expression, were detected between both strains under study. Additionally, as far as we could know, there is no currently evidence of genetic EPS regulation by this type of proteins in Gram-positive bacteria. Finally, the *eps* cluster from the strain A1dOxR, which was sequenced in a previous work (21), showed an identical structural organization, with a few nucleotide changes, but no deletions or insertions, to that found in other available *B. animalis* subsp. *lactis* genomes (23). This fact is not surprising due to the scarce genetic variability detected in the whole genomes from strains of this subspecies (24).

In short, in this study we have found an association between the high rhamnose content of the HMW-EPS synthesized by the strain A1dOxR and the over-expression of the rhamnose-biosynthesis genes located within its *eps* cluster. Further work is focused on the analysis of the genomes of these three isogenic strains and the SNPs variations among them in order to identify the molecular bases of the ropy phenotype.

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218 Strain-specific genotyping of *Bifidobacterium animalis* subsp. *lactis* by using single-nucleotide
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220

221 **Table 1** Growth parameters measured after 24 h incubation in MRS broth + 0.25% L-cysteine
222 (MRSC) cultured with the exopolysaccharide (EPS)-producing strains *Bifidobacterium animalis*
223 subsp. *lactis* A1, A1dOx and A1dOxR.

	Mean ± SD		
	Strain A1	**Strain A1dOx**	**Strain A1dOxR**
Strain phenotype	non-ropy	non-ropy	ropy
EPS MW distribution [a]	LMW	LMW	LWM + HMW
OD_{600nm}	7.97±0.11	8.60±0.11	8.27±0.13
Counts (Log cfu ml^{-1})	9.42±0.05	9.39±0.05	9.58±0.01
pH	4.36±0.01	4.39±0.01	4.41±0.01
Total organic acids (mM)	116.8±15.8	110.93±14.5	106.65±16.96
Ratio acetic/lactic acid	1.32±0.04	1.32±0.07	1.29±0.08

224 [a] Abbreviations: MW, molecular weight; LMW, low molecular weight; HMW, high molecular
225 weight

Figure legends

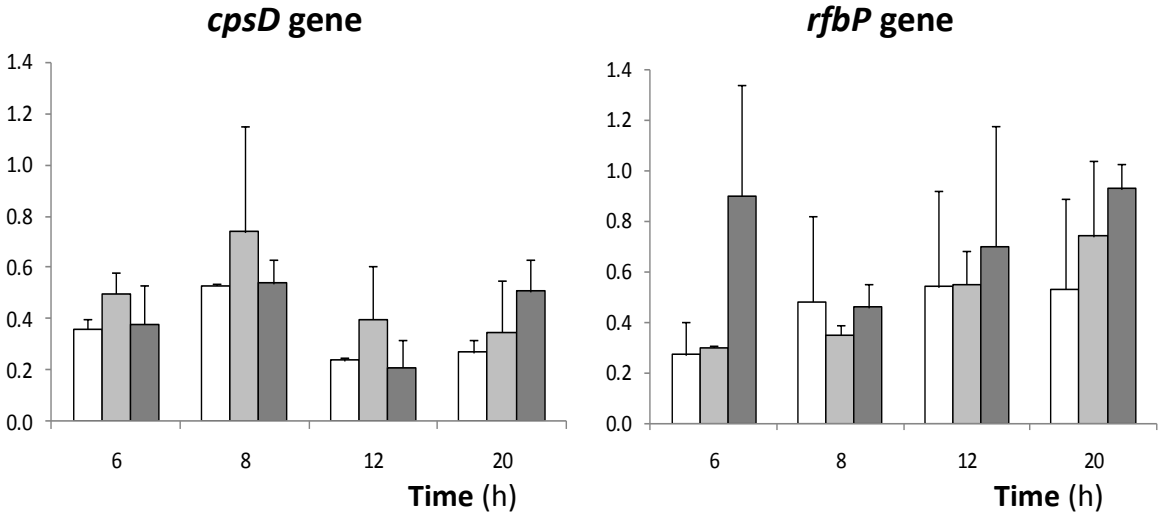
Figure 1 (A) Relative expression of *cpsD* and *rfbP* genes, encoding for the priming glycosyltransferase (GTF) CpsD and RfbP, in the EPS-producing strains *B. animalis* subsp. *lactis* A1 (white bar), A1dOx (light-grey bar) and A1dOxR (dark-grey bar) during growth in MRSC medium. Within each strain, the qPCR result of each gene was referred to that of the reporter gene *recA*, encoding for recombinase A, and finally to the basal (time 0 h) expression level. No statistical differences, analyzed by means of ANOVA tests, were detected among strains at each incubation point. **(B)** EPS production, expressed as the relative fluorescence emitted after EPS labeling with concanavalin A-Alexa Fluor 488 conjugated, by the strains *B. animalis* subsp. *lactis* A1 (white bar), A1dOx (light-grey bar) and A1dOxR (dark-grey bar) along the growth in MRSC medium. Values of fluorescence emitted were corrected by the number of bifidobacteria obtained at each sampling point and, finally, the relative fluorescence was calculated with respect to the initial point (0 h). Results represent the average values of three biological replicates which were analysed by means of one-way ANOVA tests (* $p<0.05$, ** $p< 0.01$). Bars that do not share the same letter are significantly different ($p< 0.05$) according to the mean comparison LSD test.

Figure 2 (**A**) Representative overly image of 2D-DIGE gel containing proteins extracted at middle exponential growth phase (~6 h) from the *B. animalis* subsp. *lactis* strains A1dOx (green dyed) and A1dOxR (red dyed). Protein spot appearing in yellow showed no differences in abundance between both strains, whereas those in red were more abundant in strain A1dOxR and those in green were more abundant in strain A1dOx. The numbered spots were excised from a duplicated 2D-PAGE gel stained with Gelcode™ Blue Safe and the proteins were identified by MALDI-TOF as indicated in the table. (**B**) Partial physical map of the *eps* cluster described in *B. animalis* subsp. *lactis* DSM10140 (type strain) showing some genes of interest in this work. Balat_1392: galactosyl transferase (CpsD), Balat_1978: dTDP-D-glucose 4,6-dehydratase (RfbB), Balat_1977: dTDP-4-dehydro-rhamnose 3,5-epimerase (RfbC), Balat_1976: dTDP-glucose pyrophosphylase or glucose-

252 1-phosphate thymidylytransferase (G1P_TT_ short), Balat_1971: UDP-phosphate sugar

253 phosphtransferase (RfbP).

(A) **Priming-GTF** (relative expression)



(B) **EPS production** (relative fluorescence)

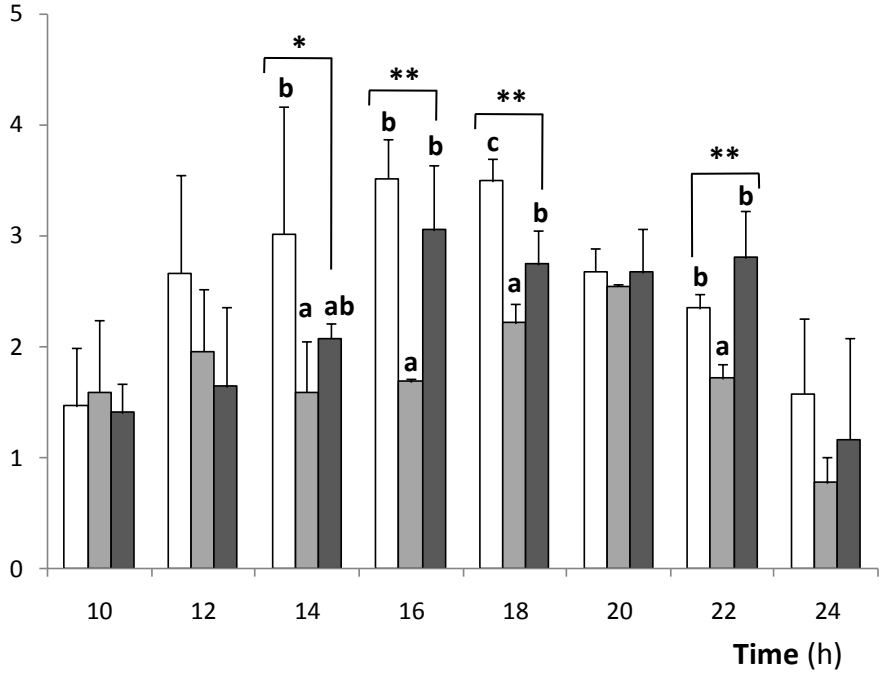
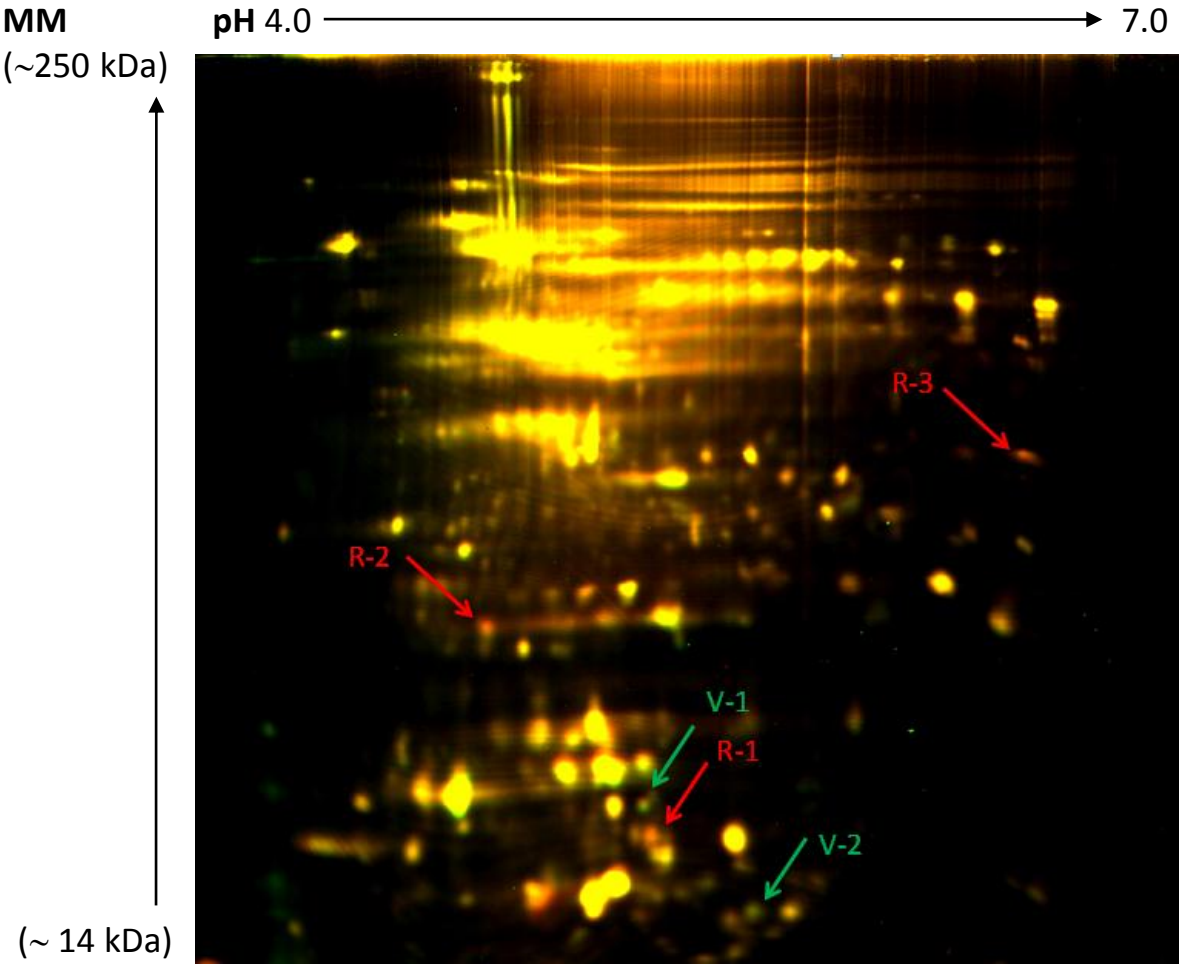


Figure 1

(A)



Spot	Predicted function	Accession no.	MOWSE[1]	Coverage	Strains[2]	
					A1dOx	A1dOxR
R1	30S ribosomal protein S6	ACS48411	121	14		R
R2	peptidyl-prolyl cis-trans isomerase	ACS48013	118	35		R
R3	dTDP-glucose 4,6-dehydratase	ACS48294	238	40		R
V1	peptidyl-prolyl cis-trans isomerase	ACS47936	96	60	G	
V2	DNA-binding protein HB1	ACS47582	131	26	G	

[1] MOWSE score resulting from the ion MS/MS search against the non redundant NCBI protein database. All scores are statistically significant ($p < 0.05$)

[2] R (red), G (green), indicates the presence of the spot only in the corresponding strain

(B)

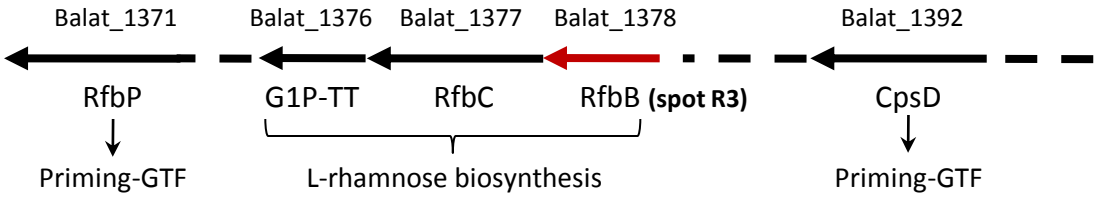


Figure 2